

(19)



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

ADL



(11)

**EP 1 138 208 A1**

(12)

**EUROPEAN PATENT APPLICATION**  
published in accordance with Art. 158(3) EPC

(43) Date of publication:  
04.10.2001 Bulletin 2001/40

(51) Int Cl.7: **A23K 1/16**

(21) Application number: **00961209.4**

(86) International application number:  
**PCT/JP00/06528**

(22) Date of filing: **22.09.2000**

(87) International publication number:  
**WO 01/22833 (05.04.2001 Gazette 2001/14)**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU  
MC NL PT SE**

- YONEDA, Hisashi Nippon Mitsubishi Oil Corp.  
Yokohama-shi Kanagawa 231-0815 (JP)
- MIZUTA, Haruyoshi Nippon Mitsubishi Oil Corp.  
Yokohama-shi Kanagawa 231-0815 (JP)

(30) Priority: **30.09.1999 JP 27933799**

(71) Applicant: **Nippon Mitsubishi Oil Corporation**  
Minato-ku, Tokyo 105-8412 (JP)

(74) Representative: **Khoo, Chong-Yee et al.**  
D Young & Co,  
21 New Fetter Lane  
London EC4A 1DA (GB)

(72) Inventors:  
• **TSUBOKURA, Akira Nippon Mitsubishi Oil Corp.**  
Yokohama-shi Kanagawa 231-0815 (JP)

**(54) PIGMENT-CONTAINING MATERIALS TO BE ADDED TO FEEDS**

(57) This invention provides a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains a high concentra-

tion of carotenoid compounds. This pigment-containing substance for feed additives is resistant to oxygen, light and so on, and can be stably conserved for a long time period.

EP 1 138 208 A1

## Description

### Technical Field

[0001] The present invention relates to a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains carotenoid compounds.

### Background Art

[0002] "Carotenoid compounds" is a generic name for a group of pigment which have a long-chain polyene structure, the majority of which have a C40 tetraterpene, and present yellow, orange, red or purple color. More specifically it refers to the compounds such as  $\beta$ -carotene, astaxanthin, canthaxanthin, zeaxanthin, echinenone, adonirubin and adonixanthin. These compounds can be used as natural pigments useful for feed additives, food additives, pharmaceuticals etc. For example, astaxanthin is valuable from an industrial point of view as a feed additive such as a color improver for bred fishes such as salmon, trout, red sea bream etc., and also as a safe natural food additive. Adonixanthin is as promising as astaxanthin, as a feed additive, a food additive and pharmaceutical as the astaxanthin is, if its industrial production process can be established. Canthaxanthin has been used as a feed additive, food additive, cosmetic, etc., and zeaxanthin has been used as a feed additive and food additive. Further, echinenone and adonirubin also expected to be used as feed additives, food additives etc. As production processes for these carotenoid compounds, chemical synthesis, production by microorganisms, extraction from natural products etc. are known, and regarding astaxanthin and canthaxanthin, their chemical synthetic products are already on sale. Comparing with a chemical synthesis, the production process of carotenoid compounds by microorganisms is advantageous due to the high level of safety, since neither metal catalysts nor solvents are used therein. Generally, carotenoid compounds are unstable in the presence of oxygen and light. However, since carotenoid compounds produced by microorganisms are accumulated inside the cell of a microorganism, their stability is ensured by the cell membrane, cell wall and other antioxidants. When carotenoid compounds are used as feed additives or the like, a high level of storage stability is an advantage, and if the microorganism itself can be used, this would be a great advantage. In contrast, if intercellular carotenoid compounds are used after being extracted and purified, it is necessary to use chlorine-containing solvents or the like which may be unsafe. Accordingly, the use of a microorganism culture precipitate consisting of a microorganism which contain carotenoid compounds as a feed additive has a great advantage. In the case where the microorganism producing carotenoid compounds has a hard cell wall, the absorption efficiency is extremely low if a natural cell is

provided. So the cell should be disrupted mechanically or decomposed with chemical agents or enzymes, and this leads to a cost disadvantage. In addition, there is a problem that the stability of carotenoid compounds decreases after the cell is disrupted. Furthermore, when the content of carotenoid compounds existing in a microorganism culture precipitate is low, a large amount of precipitate is needed in the production process of the feed, and this causes other problems such as poor operability, high transport cost and nutritional imbalance of the feed. However, there is not known a pigment-containing substance consisting of a microorganism whose lack of a hard cell wall allows easy use of the accumulated carotenoid compounds; and, further consisting of a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.

[0003] Among carotenoid compounds, astaxanthin is a red pigment contained in Pisces such as salmon, trout, red sea bream etc., and Crustacea such as shrimps, crabs, etc., and it is useful because of its beautiful color, and as stated above, it has been broadly used as a feed additive and a natural food additive. Regarding the red yeast *Phaffia rhodozyma*, which is known to produce astaxanthin, there is a problem that, being a yeast, it has a hard cell wall. As processes for the production of astaxanthin by bacteria, the followings are known, but regarding each of them, the content per weight of dry cell is low: a bacterium *Brevibacterium* No. 103 strain belonging to the genus *Brevibacterium* produces merely 0.003% of astaxanthin per weight of dry cell (*Journal of General and Applied Microbiology*, 15, 127; 1969). Also, another bacterium *Paracoccus marcusii* DSM11574 strain belonging to the genus *Paracoccus* produces only 0.022% of astaxanthin per weight of dry cell (WO 99/6586).

[0004] The object of the present invention is to provide a pigment-containing substance for feed additives consisting of a microorganism culture precipitate which contains a high concentration of carotenoid compounds useful as a natural pigment.

### Disclosure of the Invention

[0005] In order to achieve the above object, the present invention provides the following means.

1. A pigment-containing substance for feed additives comprising a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.
2. The pigment-containing substance for feed additives, characterized in that at least 40 mass % of the carotenoid compounds is astaxanthin.
3. The pigment-containing substance for feed additives, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the

microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence shown in SEQ ID NO: 1.

4. The pigment-containing substance for feed additives, characterized in that the microorganism in the microorganism culture precipitate is E-396 strain or a mutant, thereof.

[0006] This specification includes part or all of the contents disclosed in the specification of Japanese Patent Application No. 11-279337, which is a priority document of the present application.

[0007] The present invention is described more specifically as follows.

[0008] The pigment-containing substance for feed additives of the present invention comprises a microorganism culture precipitate. First, the microorganism culture precipitate is disclosed. The microorganism culture precipitate of the present invention can be obtained by a process wherein mainly the cell of microorganism producing carotenoid compounds is cultured to produce these compounds, the resulting culture is treated by filtration, centrifugation etc., followed by the removal of a certain amount of moisture. Any method for culturing a strain which produces carotenoid compounds can be used on the condition that carotenoid compounds are generated. For example, the following method can be adapted. As a medium, one which contains the followings is used: carbon sources, nitrogen sources, inorganic salt, which are necessary for the growth of bacteria producing carotenoid compounds, and particularly required substances (e.g. vitamins, amino acids, nucleic acid bases etc.) as needed. The carbon sources to be used include carbohydrate such as glucose, sucrose, fructose, trehalose, mannose, mannitol and maltose; organic acids such as acetic acid, fumaric acid, citric acid, propionic acid, malic acid and malonic acid; alcohols such as ethanol, propanol, butanol, pentanol, hexanol and isobutanol; and the like. The ratio of added carbon sources depends on the type of carbon sources, but generally 1 to 100g per L of medium, preferably 2 to 50g is applied. The nitrogen sources to be used include one or more selected from potassium nitrate, ammonium nitrate, ammonium chloride, ammonium sulfate, diammonium hydrogen phosphate, ammonia, urea and the like. The ratio of added nitrogen sources depends on the type of nitrogen sources, but generally 0.1 to 10g per L of medium, preferably 1 to 3g is applied. The inorganic salt to be used includes one or more selected from potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium phosphate, magnesium sulfate, magnesium chloride, ferrous sulfate, ferrous chloride, manganous sulfate, manganous chloride, zinc sulfate, zinc chloride, cupric sulfate, calcium chloride, calcium carbonate, sodium carbonate and the like. The ratio of added inorganic salt depends on the type of inorganic salt, but generally 0.001 to 10g per L of medium are ap-

plied. The particularly required substances to be used include one or more selected from vitamins, nucleic acids, yeast extract, peptone, meat extract, malt extract, corn steep liquor, dry yeast, soybean cake, soybean oil, olive oil, corn oil, linseed oil and the like. The ratio of added particularly required substances depends on the type of the substances, but generally 1 to 200g per L of medium, preferably 10 to 100g is applied. The pH of the medium is 2-12, preferably it is adjusted to 6-10. As culture conditions, a shaking culture or an aeration agitation culture is carried out at 15-80°C, preferably at 20-35°C, ordinarily for 1-20 days, preferably for 2-8 days.

[0009] Next, moisture is removed from the culture obtained by the above method. The amount of moisture removed to obtain the pigment-containing substance for feed additives of the present invention depends on the state of the cultured medium (e.g. pigment content), but as a general process, first filtration is carried out; and when removal of moisture is further needed, drying of the precipitate follows. The methods of filtration include ordinary filtration, centrifugation and the like. Since the obtained precipitate contains water and precipitate comprising dissolved medium ingredients such as saline and carbohydrate, it is effective that water is added to the precipitate removed from the cultured medium, suspended, and then the precipitate is separated the precipitate again, so that the amount of carotenoid compounds therein increases. By this process, the medium ingredients dissolved in water can be removed to some extent. In the case where the amount of carotenoid compounds should be increased, it is possible to apply a method for removing moisture by drying the precipitate. The methods for drying the precipitate include ordinary spray drying, drum drying, freeze drying and the like.

[0010] The microorganism culture precipitate obtained by the above method can be used successfully as a pigment-containing substance. For the purpose of preventing the decomposition of carotenoid compounds, antioxidants such as BHT (butylated hydroxytoluene), ethoxyquin and vitamin E may be added to the culture precipitate. Further, the surface of the microorganism may be covered with gelatin or the like.

[0011] Now, the microorganism used for the present invention is described. The microorganism used for the present invention is not specifically limited so long as it can produce carotenoid compounds by the culture of microorganisms such as bacteria and yeast, and can contain at least 3 mass % carotenoid compounds in its culture precipitate. However, considering the use of carotenoid compounds accumulated in the microorganism during the culture, it is preferable to employ a bacterium whose cell wall is thin enough to utilize the pigment effectively. In view of the growth speed and productivity of carotenoid compounds, it is especially preferable that a DNA nucleotide sequence corresponding to 16S ribosomal RNA is substantially homologous with the nucleotide sequence shown in SEQ ID NO: 1.

[0012] Taking the frequency of occurrence of errors in determining DNA nucleotide sequence into consideration, the term "substantially homologous" used herein means at least 98% homology.

[0013] In the bacteria having the sequence substantially homologous with the above sequence, carotenoid compounds such as astaxanthin, adonixanthin,  $\beta$ -carotene, echinenone, canthaxanthin, zeaxanthin,  $\beta$ -cryptoxanthin, 3-hydroxyechinenone, asteroidenone, adonirubin are accumulated by culture as a mixture. The ratio of generated carotenoid compounds contained in the cell can be changed, for example by changing aerobic culture conditions. By way of example, the ratio of generated adonixanthin can be changed by increasing the concentration of dissolved oxygen in culture medium. Also, the cell having an altered ratio of carotenoid compound production can be obtained by mutation. The methods of mutation include physical methods such as X-ray irradiation and ultraviolet ray irradiation, the use of chemical mutagens, and artificial mutations wherein a chemical method of mutation such as treatment with NTG (N-methyl-N'-nitro-N'-nitrosoguanidine) and EMS (ethylmethane sulfonate).

[0014] Among the said bacteria, E-396 strain is one wherein astaxanthin makes up at least 40 mass % of the generated carotenoid compounds. This strain was newly isolated by the present inventors, and was deposited with the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (1 Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, Japan) under accession No. FERM BP-4283 on April 27, 1993. The mycological properties of this strain are disclosed in Japanese Patent Application Laying-Open (kokai) Nos. 7-79796, 8-9964 and 9-308481. A DNA nucleotide sequence corresponding to 16S ribosomal RNA of this strain is as shown in SEQ ID NO: 1.

[0015] Astaxanthin produced by the strain whose DNA nucleotide sequence corresponding to 16S ribosomal RNA is substantially homologous with the sequence specified by the present invention, is (3S, 3'S)-astaxanthin, and the purity is almost 100%. Astaxanthin presented in natural products such as crayfish, haematococcus, salmon, trout and red sea bream is known to contain a high rate of (3S, 3'S). In contrast, *Phaffia rhodozyma* is known to contain a high rate of (3R, 3'R) which has an absolute configuration which is the reverse of astaxanthin presented in the nature. The astaxanthin produced by the strain of the present invention is 100% (3S, 3'S)-astaxanthin and it is valuable from an industrial point of view that it has the same absolute configuration as the majority of astaxanthins in nature.

[0016] Some examples are provided below to describe the present invention more specifically, but the present invention is not limited thereto.

## Best Modes for Carrying Out the Invention

### Example 1:

[0017] A medium 6mL consisting of the composition of Table 1 was put into a test tube whose diameter is 18mm and a steam sterilization was carried out at 121°C for 15 minutes. One platinum loop of E-396 strain (FERM BP-4283) was inoculated thereto, and a reciprocal shaking culture was carried out at 350 rpm at 28°C for 2 days. 2mL of this culture was transferred to a 500 mL Sakaguchi flask containing 100mL of the medium which has the same composition as the above, and a reciprocal shaking culture was carried out at 100 rpm at 28°C for 6 days. Cells (wet weight 3.2g) were obtained from 100mL of the cultured medium by centrifugation. After the cells were added to 50 mL of ion-exchange water and fully suspended and centrifugation was carried out again to obtain cells (wet weight 3.1g). Then, 3.1g of the cells was freeze-dried to obtain 1.1g of dried cell. The carotenoid content existing in the dried cell was analyzed by high-performance liquid chromatography. The composition of carotenoid compounds is shown in Table 2. The water content in the dried cell was 2.5%.

Table 1.

Composition	Added amount (g/L)
Yeast extract	20
Peptone	5
Sucrose	100
$\text{KH}_2\text{PO}_4$	1.5
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	3.8
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.01
pH7 (adjusted to this value using $\text{Na}_2\text{CO}_3$ )	

Table 2.

Carotenoid compounds	Content (mg/g)
$\beta$ -carotene	1.6
echinenone	1.9
3-hydroxyechinenone	0.9
canthaxanthin	2.3
adonirubin	5.6
astaxanthin	13.0
asteroidenone	0.6
adonixanthin	5.3
zeaxanthin	0.01
total carotenoid	31.2

### Example 2:

[0018] E-396 strain (FERM BP-4283) was mutated

with NTG (N-methyl-N'-nitro-N-nitrosoguanidine) and intensely red colored colonies were selected. Carotenoid compounds in the cultured medium were analyzed, and mutant strains with increased astaxanthin yield were selected. The medium 5mL consisting of the composition of Table 1 was put into a test tube whose diameter is 18mm and a steam sterilization was carried out at 121°C for 15 minutes. One platinum loop of the mutant E-396 strain was inoculated thereinto, and a reciprocal shaking culture was carried out at 300 rpm at 30°C for 2 days. 2mL of this culture was transferred to a 500mL Sakaguchi flask containing 100mL of the medium which has the same composition as the above, and a reciprocal shaking culture was carried out at 120 rpm at 29°C for 2 days. Then, 800mL of this culture was inoculated into a 30L fermenter containing 20L of the medium consisting of the composition of Table 3, and an aerobic fermentation was carried out at 400rpm at 1.0vm at 29°C for 150 hours. Cells (wet weight 600g) were obtained from 18L of the cultured medium by Sharples centrifuge. After the cells were added to 20L of tap water and fully suspended, cells (wet weight 530g) were obtained by again using the same Sharples centrifuge. Then, after 1.5L of tap water was added and the cells (wet weight 530g) were fully suspended; and the cell was dried with a spray drier to obtain 200g of dried cell. As operating conditions, the air temperature was 210°C when introduced and was 105°C at exit, and the rate at which the suspension was fed was 38mL/min. The carotenoid content existing in the dried cell was analyzed by high-performance liquid chromatography. The composition of carotenoid compounds is shown in Table 4. The water content in the dried cell was 3.1%.

Table 3

Composition	Added amount (g/L)
Yeast extract	20
Peptone	5
glucose	120
KH <sub>2</sub> PO <sub>4</sub>	1.5
Na <sub>2</sub> HPO <sub>4</sub> · 12H <sub>2</sub> O	3.8
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.5
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.01
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.01
pH7 (adjusted to this value using Na <sub>2</sub> CO <sub>3</sub> )	

Table 4

Carotenoid compounds	Content (mg/g)
β-carotene	0.7
echinenone	1.1
3-hydroxyechinenone	0.6
canthaxanthin	1.7
adonirubin	5.4

Table 4 (continued)

Carotenoid compounds	Content (mg/g)
astaxanthin	19.4
asteroidenone	0.8
adonixanthin	5.5
zeaxanthin	0.02
total carotenoid	35.2

#### Advantage of the Invention

[0019] Since the carotenoid existing in the pigment-containing substance for feed additives of the present invention is stabilized by the action of cell membrane, cell wall and the like of a microorganism, the pigment-containing substance for feed additives is resistant to oxygen, light and so on, and can be stably stored for a long time period. All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

#### Claims

1. A pigment-containing substance for feed additives comprising a microorganism culture precipitate which contains at least 3 mass % carotenoid compounds.
2. The pigment-containing substance for feed additives according to claim 1, characterized in that at least 40 mass % of the carotenoid compounds is astaxanthin.
3. The pigment-containing substance for feed additives according to claim 1 or 2, characterized in that a DNA nucleotide sequence corresponding to 16S ribosomal RNA of the microorganism in the microorganism culture precipitate has at least 98% homology with the nucleotide sequence as shown in SEQ.ID.NO: 1.
4. The pigment-containing substance for feed additives according to claim 3, characterized in that the microorganism in the microorganism culture precipitate is E-396 strain or a mutant thereof.

EP 1 138 208 A1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06528

A. CLASSIFICATION OF SUBJECT MATTER  
Int.Cl.<sup>7</sup> A23K 1/16

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.<sup>7</sup> A23K 1/16, C12P 23/00, C12N 1/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
WPIDS, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP, 9-308481, A (NIPPON OIL COMPANY, LTD.), 02 December, 1997 (02.12.97) (Family: none)	1-4
X	EP, 635576, A1 (Nippon Oil KK), 25 January, 1995 (25.01.95) & JP, 7-79796, A & NO, 9402731, A & CA, 2128549, A & US, 5607839, A & US, 5858761, A & EP, 635576, B1	1-4
A	EP, 747483, A2 (Hoffmann la Roche & CO AG F), 11 December, 1996 (11.12.96) & JP, 9-23888, A & US, 6087152, A & US, 6124113, A & CN, 1141952, A	1-4
A	JP, 6-165684, A (Kaiyo Bio Technology Kenkyusho K.K.), 14 June, 1994 (14.06.94) (Family: none)	1-4
A	JP, 5-328979, A (Kaiyo Bio Technology Kenkyusho K.K.), 14 December, 1993 (14.12.93)	1-4
A	EP, 454024, A (Phillips Petroleum CO), 30 October, 1991 (30.10.91)	1-4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not

considered to be of particular relevance

"E" earlier document but published on or after the international filing

date

"L" document which may throw doubts on priority claim(s) or which is

cited to establish the publication date of another citation or other

special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other

means

"P" document published prior to the international filing date but later

than the priority date claimed

"T" later document published after the international filing date or

priority date and not in conflict with the application but cited to

understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be

considered novel or cannot be considered to involve an inventive

step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be

considered to involve an inventive step when the document is

combined with one or more other such documents, such

combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search  
26 December, 2000 (26.12.00)

Date of mailing of the international search report  
16 January, 2001 (16.01.01)

Name and mailing address of the ISA/  
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

EP 1 138 208 A1

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/06528

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& NO, 9101577, A & AU, 9173639, A & CA, 2033666, A & FI, 9101948, A & JP, 4-228064, A	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)